

Original Article



The Effects of Sodium Metabisulfite on the Hippocampus and Prefrontal Cortex in Wistar Rats: A Cognitive, Neurochemical, and Histological Study

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Article info:

Received: 13 Jul 2023

Accepted: 17 Nov 2023

Keywords:

Sodium metabisulfite,
Hippocampus,
Prefrontal cortex,
Neurodegeneration,
Acetylcholinesterase,
Cognition

ABSTRACT

Background: Studies have demonstrated that sub-chronic exposure to sodium metabisulfite (NaMBS) can have detrimental impacts on animal brain structure and function. However, there is scant research regarding its effect on certain brain regions and cognitive effects.

Objectives: This study investigates the potential effect of sub-chronic exposure to NaMBS on the hippocampus and prefrontal cortex of female Wistar rats.

Methods: A total of 24 adolescent female Wistar rats were randomly divided into four groups (6 rats each) as follows: Group 1 (control) received 0.5 mL of normal saline; group 2 was administered by 100 mg/kg of NaMBS; group 3 was issued by 300 mg/kg of NaMBS; group 4 received 500 mg/kg of NaMBS. The route of administration was oral for 28 days. After completing the administration phase, the Y-maze test was conducted. Subsequently, the rats were euthanized, and tissue samples from the prefrontal cortex and hippocampus were collected for biochemical assays, precisely measuring malondialdehyde (MDA) and acetylcholinesterase (AChE) levels, as well as histological studies, such as hematoxylin and eosin, and glial fibrillary acidic protein (GFAP). Meanwhile, the neuronal count was done.

Results: The learning and memory functions of rats in the treated and control groups were similar (number of alternations: $P > 0.05$). The group treated with 500 mg/kg NaMBS presented indications of neurodegeneration in CA1 of the hippocampus and high glial fibrillary acidic protein-immunoreactivity but had no noticeable effects on layer II/III of the prefrontal cortex. Regardless of the dosage of NaMBS, malondialdehyde level was the same ($P > 0.05$) for all groups; however, in the group that received 500 mg/kg NaMBS, the acetylcholinesterase level was significantly reduced ($P < 0.05$).

Conclusion: This study revealed that while NaMBS can lead to neurodegeneration in the hippocampus of Wistar rats at 500 mg/kg, the prefrontal cortex remains resilient and spatial memory is unaffected, but a decrease in acetylcholinesterase activity raises cognitive concerns, emphasizing the need for cautious consideration of its use.

Citation Amedu NO, Abolarin P, Wilcox F, Abdur-Rahman H. The Effects of Sodium Metabisulfite on the Hippocampus and Prefrontal Cortex in Wistar Rats: A Cognitive, Neurochemical, and Histological Study. *Pharmaceutical and Biomedical Research*. 2024; 10(1):11-22. <http://dx.doi.org/10.32598/PBR.10.1.1065.2>

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Introduction

Sodium metabisulfite (NaMBS) is employed in the food, beverage, cosmetic, medical, rubber, and photographic sectors, functioning as a preservative, bleaching, and antioxidative agent [1]. It holds significant importance as a preservation method for various items, such as wine, beer, grapefruit juice, dried fruits, and seafood. It has widespread usage in the production of processed foods [2]. In certain circumstances, sodium metabisulfite exhibits properties that counteract peroxide effects, creating harmful oxidizing agents [3].

NaMBS demonstrates diverse impacts. For instance, higher doses and prolonged exposure to NaMBS are linked to reduced root growth, decreased mitotic index, and increased chromosomal abnormalities [4]. NaMBS can potentially induce significant detrimental effects on healthy human cells through various mechanisms, including initiating apoptosis [5]. Consumption of sub-chronic amounts of sodium metabisulfite leads to changes in immune response and shifts in biochemical, hematological, and physiological parameters in Wistar rats [6].

Malondialdehyde (MDA) concentrations exhibited a notable rise in rats subjected to NaMBS treatment [7]. Similarly, NaMBS triggers lipid peroxidation and prompts apoptosis in rats [8]. Regarding its impact on potassium currents in isolated CA1 pyramidal neurons of the rat hippocampus, NaMBS displayed a concentration-dependent elevation in transient outward and delayed rectifier potassium currents [9]. The influence of NaMBS on apoptosis within the experimental model of Parkinson disease suggests that sulfite does not exacerbate the activity of caspase-3 in the context of a 6-hydroxydopamine-induced Parkinson disease model [10]. According to a study exploring the effects of NaMBS on locomotor activity in the Parkinson disease experimental model, sulphite does not aggravate the activity of cyclooxygenase or the levels of prostaglandin E2 or nuclear factor kappa B in a 6-hydroxydopamine-induced Parkinson disease model [11]. Sodium metabisulfite is associated with neuronal toxicity due to its ability to heighten neuronal excitability [12].

The hippocampus, the prefrontal cortex, and interconnected networks of neurons play roles in various aspects of cognitive and memory functions. The hippocampus-prefrontal pathway innervates the prelimbic/medial orbital areas of the prefrontal cortex in the rat [13]. Previous studies have reported the effects of NaMBS on

specific regions of a rat's brain. However, these effects are not gender specific. For example, NaMBS was said to have induced structural changes in the medial prefrontal cortex [1]. Meng and Nie [9] reported that NaMBS increased potassium and sodium currents in rat hippocampal neurons, potentially decreasing their excitability. Furthermore, Lai et al. [14] reported that NaMBS stimulated sodium currents and increased cellular excitability in excitable cells. Cumulatively, prior research has demonstrated that sub-chronic exposure to NaMBS can have intricate and potentially detrimental impacts on animal brain structure and function. However, a deficiency or scarcity of data exists regarding certain brain regions and cognitive effects. Additionally, there is a limited understanding of the gender-specific effects of NaMBS on specific brain regions. Recognizing the observed gaps in knowledge, this study explores the potential effects of sub-chronic NaMBS exposure on the hippocampus and prefrontal cortex in female Wistar rats.

Materials and Methods

Study animals

A total of 24 adolescent female Wistar rats (mean weight of 206.7 g) were acquired from Temilola Animal Husbandry in Ogbomoso, Oyo State, Nigeria. These rats were accommodated within the Faculty of Basic Medical Sciences animal facility at Adeleke University in Ede. The rats were housed in conventional plastic rat cages equipped with iron lids. A 7-day acclimatization period was observed before categorization and the initiation of substance administration. The rats were maintained under consistent, equal dark-light cycles throughout this acclimatization period and until the experiment's conclusion. Additionally, the rats were provided unrestricted access to food and water, allowing them to consume as desired.

Experimental design

Following the acclimatization phase, the rats were allocated randomly into four sets, each containing six rats. Over 28 consecutive days, the treatments were administered through oral gavage in the following manner: Group 1 (control) was given 0.5 mL of normal saline; group 2 received NaMBS at a dosage of 100 mg/kg of body weight; group 3 was administered NaMBS at a dosage of 300 mg/kg of body weight; and group 4 received NaMBS at a dosage of 500 mg/kg of body weight.

Behavioural tests

The behavioral assessments took place from 8:00 AM to 12:00 PM, beginning a day after the conclusion of the treatment session.

Y-maze test

The Y-maze test evaluates rodents' spatial working and reference memory, capitalizing on their inherent exploratory behavior. The testing procedure was based on a modified approach outlined by Kraeuter et al. [15]. This method employed a maze with three arms designated as A, B, and C. Throughout the test, the rats were observed for 5 min while they freely navigated the maze. Working memory was evaluated by counting the instances of alternations, which refer to successive entries into all three arms without revisiting the same arm consecutively. Following the test, video analysis was conducted to tally the number of arm entries and alternations, enabling the computation of alternation frequency. A heightened alternation frequency indicated enhanced memory performance, signifying that the rat retained recollection of previously explored arms.

Tissue collection

Following the conclusion of the behavioral assessment, the rats designated for histopathological analysis were anesthetized through intraperitoneal injection of ketamine (50 mg/mL, Pakson Pharma, PVT Ltd., India). Subsequently, perfusion fixation was carried out using 4% paraformaldehyde (PFA), aligning with the protocol outlined by Gage et al. [16]. The rats were decapitated, and the hippocampi and prefrontal cortex were meticulously removed from both hemispheres. These excised brain regions were subjected to a post-fixation process in 4% PFA for 18 h.

For the neurochemical analyses, the rats were humanely euthanized through cervical dislocation, followed by decapitation. This method of euthanasia was selected to prevent any potential impact of anesthetic agents on the specific neurochemicals under investigation. The hippocampi and prefrontal cortices from both hemispheres were carefully removed and homogenized in a 0.1 M phosphate buffer with a pH of 8.0, employing a Teflon Potter-Elvehjem homogenizer. The resultant homogenates underwent centrifugation at 4000 rpm for 10 min. Subsequently, the supernatants were separated and transferred into test tubes to assess MDA and acetylcholinesterase (AChE) levels.

Malondialdehyde assay

In this procedure, MDA H_3PO_4 (1%, 3 mL) and aqueous thiobarbituric acid solution (0.6%, 3 mL) were combined with a 10% homogenate (0.5 mL). The mixture underwent shaking and was heated in a boiling-water bath for 45 min. After cooling, n-butanol (4 mL) was added, and the mixture was shaken again. Centrifugation at 1200 g for 15 min separated the n-butanol layer, and its optical density was measured using a spectrophotometer, utilizing absorption wavelengths of 520 nm. The concentration of MDA was calculated and expressed as μmol MDA per gram of wet tissue.

Acetylcholinesterase assay

The activity of AChE was assessed by employing the colorimetric method outlined in the work of Ellman et al. [17]. The absorbance measurement was taken at a wavelength of 412 nm, and the absorbance was quantified in units of nmol/L.

Histopathological study

Following an 18-h post-fixation in 4% PFA, the tissue specimens underwent processing and were embedded in paraffin blocks. These tissue blocks were then cut into sections and subjected to hematoxylin and eosin (H&E) staining, following the protocols outlined by Bancroft and Gamble [18], to perform standard histological analysis of the hippocampus and prefrontal cortex. The stained tissue sections were observed using light microscopy, utilizing the Olympus model (XSZ-107 BN [New Jersey, USA] and Amscope, MD500) [19].

Immuno-histochemistry assay

Tissue sections underwent deparaffinization and antigen retrieval, followed by treatment with phosphate-buffered saline and hydrogen peroxide to block endogenous peroxidase activity. Background staining was suppressed with normal goat serum, and then sections were incubated with polyclonal rabbit anti-gial fibrillary acidic protein (GFAP) serum (diluted 1:500) for 24 h at 4°C. Biotinylated mouse anti-rabbit solution and the Avidin-Biotin complex were applied successively, followed by visualization of the immunocomplex using 3,3'-diaminobenzidine. Haematoxylin counterstaining was performed, and the sections were observed under light microscopy at a scale bar of 180 μ .

Morphometric analyses

Morphometric analyses were performed using the ImageJ software, version 1.52r, on sections stained with H&E and GFAP immunostaining. Five sections from six distinct visual fields were assessed in each tissue block, spanning magnifications ranging from 180 μ m. For H&E-stained sections, the count of pyramidal cells (PCs) within the CA1 region and layer II/III of the prefrontal cortex was conducted for all groups. Moreover, GFAP-immunoreactive astrocytes were quantified in the CA1 and dentate gyrus regions. The criteria for counting GFAP-immuno-positive cells were based on clearly identifiable cell bodies with extended processes, following the methodology outlined by a previous study [20].

Statistical analyses

Data analysis was conducted using GraphPad Prism software, version 7. The statistical comparisons involved utilizing a one-way analysis of variance in combination with the Tukey multiple comparison test. All outcomes were presented as Mean $\pm$ SD. In addition, statistical significance was determined at a threshold of $P<0.05$.

Results

Changes in learning and memory in the study groups (number of complete alternations)

The number of complete alternation from the Y-maze test (Table 1) shows no statistically significant difference ($P>0.05$) between the treated groups (100 mg/kg, 300 mg/kg, 50 mg/kg) and the control group (2.3 ± 0.4 ; 2.3 ± 0.47 ; 1.7 ± 0.4 vs 2.7 ± 0.47).

Variations in malondialdehyde and acetylcholinesterase levels

MDA concentration (Table 2) demonstrates no notable statistical distinction ($P>0.05$) between the groups subjected to treatment and the control group (0.59 ± 0.13 ; 0.64 ± 0.26 ; 0.68 ± 0.13 vs. 0.48 ± 0.04). The level of AchE (Table 2) in the group treated with NaMBS (500 mg/kg) exhibited a significant reduction ($P<0.05$) compared to the control group (26 ± 3.2 nmol/L vs 55 ± 2.5 nmol/L). When making comparisons, no substantial variance ($P>0.05$) in AchE levels was observed between the groups treated with 100 mg/kg and 300 mg/kg and the control group (Table 2).

Histopathological observations

According to Figure 1, the H&E-stained sections of CA1 reveal closely clustered PCs with regular neuronal processes in both the control and group treated with NaMBS 100 mg/kg. Conversely, the group treated with NaMBS 500 mg/kg presents indications of neurodegeneration, characterized by a notable presence of shrunken PCs. The assessment of neuronal cell count within CA1 (Figure 2) demonstrates noteworthy findings. The study group subjected to NaMBS 500 mg/kg showcases a considerably lower ($P<0.05$) count of PCs (638 ± 41) compared to the control group (750 ± 62). Additionally, the number of PCs in the CA1 region of the study groups treated with NaMBS 100 mg/kg and NaMBS 300 mg/kg exhibit no significant variations ($P>0.05$) compared to the control group (Figure 2).

Table 1. Complete alternation in the Y-maze test of the study groups (n=6)

Parameter	Mean $\pm$ SD			
	Control	NaMBS 100 mg/kg	NaMBS 300 mg/kg	NaMBS 500 mg/kg
Number of complete alternations	2.7 $\pm$ 0.4	2.3 $\pm$ 0.47	2.3 $\pm$ 0.4	1.7 $\pm$ 0.47

SD: Standard deviation; NaMBS: Sodium metabisulfite.

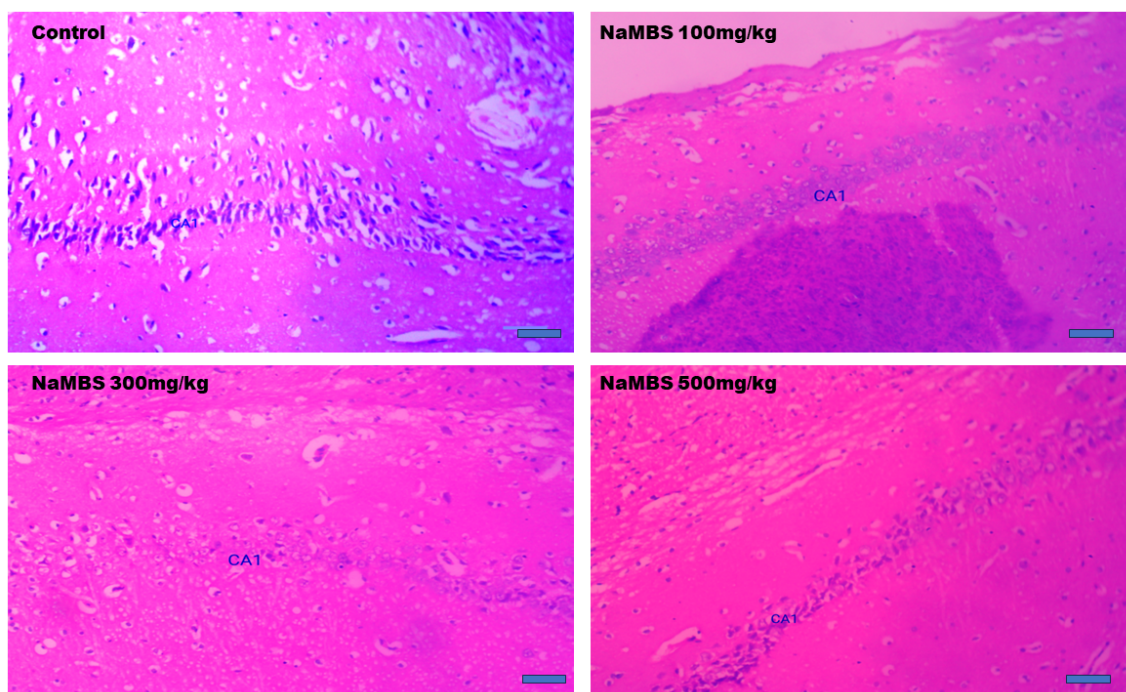
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Table 2. Changes in malondialdehyde and acetylcholinesterase levels in the brain of rats in the study groups

Parameters	Mean $\pm$ SD			
	Control	NaMBS 100 mg/kg	NaMBS 300 mg/kg	NaMBS 500 mg/kg
MDA (μ m/g)	0.48 $\pm$ 0.04	0.59 $\pm$ 0.13	0.64 $\pm$ 0.26	0.68 $\pm$ 0.13
AchE (nmol/L)	55 $\pm$ 2.5	38 $\pm$ 9.1	41 $\pm$ 7.6	26 $\pm$ 3.2 ^a

Abbreviations: SD: Standard deviation; NaMBS: Sodium metabisulfite; MDA: Malondialdehyde; AchE: Acetylcholinesterase. ^a $P<0.05$ vs control.

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Figure 1. Representative photomicrographs of hematoxylin and eosin stained sections of the hippocampus (magnification x100) CA1: Cornu ammonis.

According to [Figure 3](#), like the control group, all the treated groups exhibit appropriately organized neurons in layer II/III of the prefrontal cortex. Additionally, the assessment of neuronal count within layer II/III ([Figure 4](#)) indicates no statistically significant distinctions ($P>0.05$) between the treated groups (NaMBS 100 mg/kg, NaMBS

300 mg/kg, NaMBS 500 mg/kg) and the control group in the conducted comparisons.

According to [Figure 5](#), the GFAP-immunostained sections of the prefrontal cortex demonstrate no apparent immunoreactivity in both the control group and the NaMBS 100 mg/kg treated group; however, in the pre-

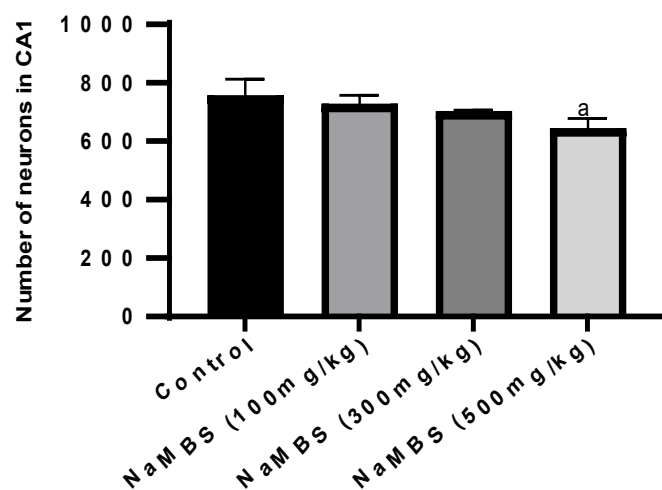
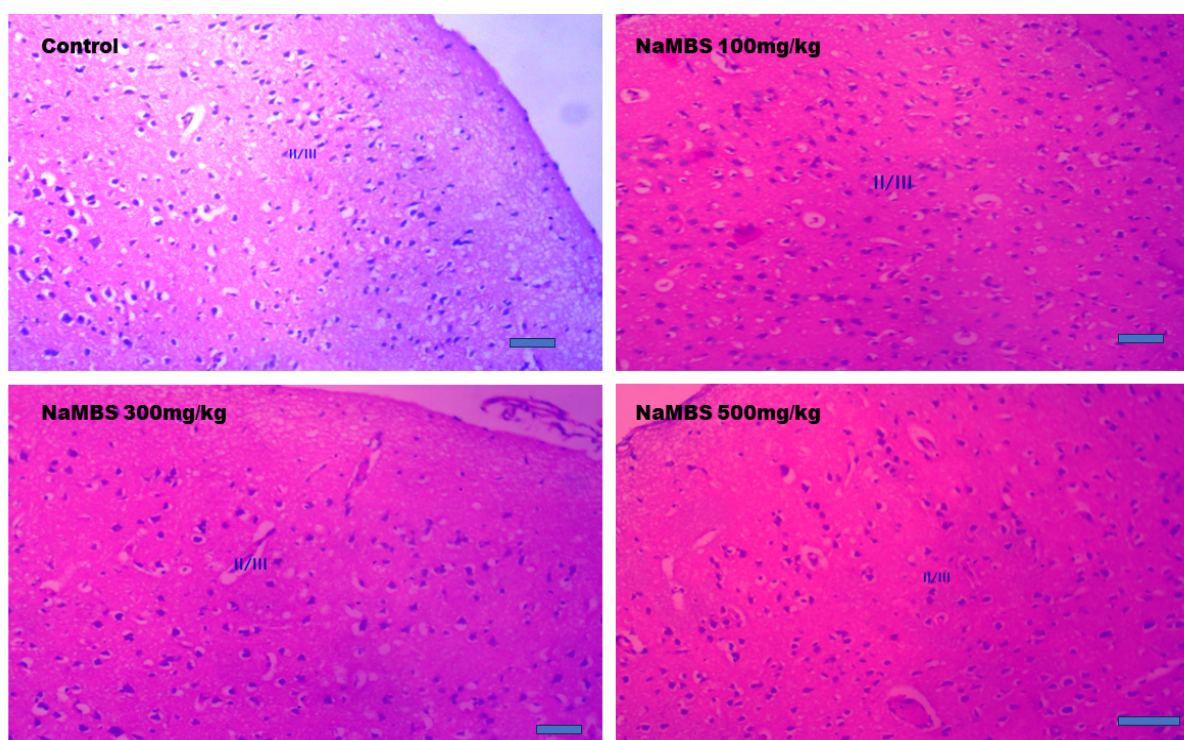


Figure 2. Number of pyramidal cells in CA1 of the hippocampus

NaMBS: Sodium metabisulfite; CA1: Cornu ammonis 1.

Notes: Values are presented as Mean±SD. ^aP<0.05 vs control.

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Figure 3. Representative photomicrographs of hematoxylin and eosin stained sections of the prefrontal cortex layer II/III (magnification x100)

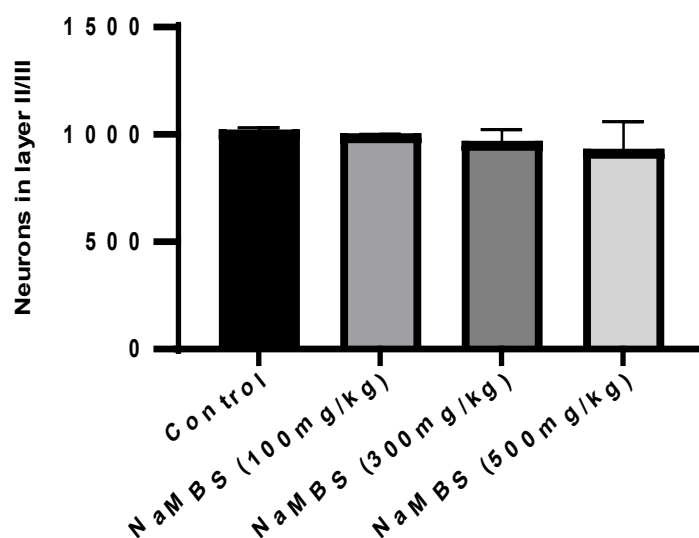
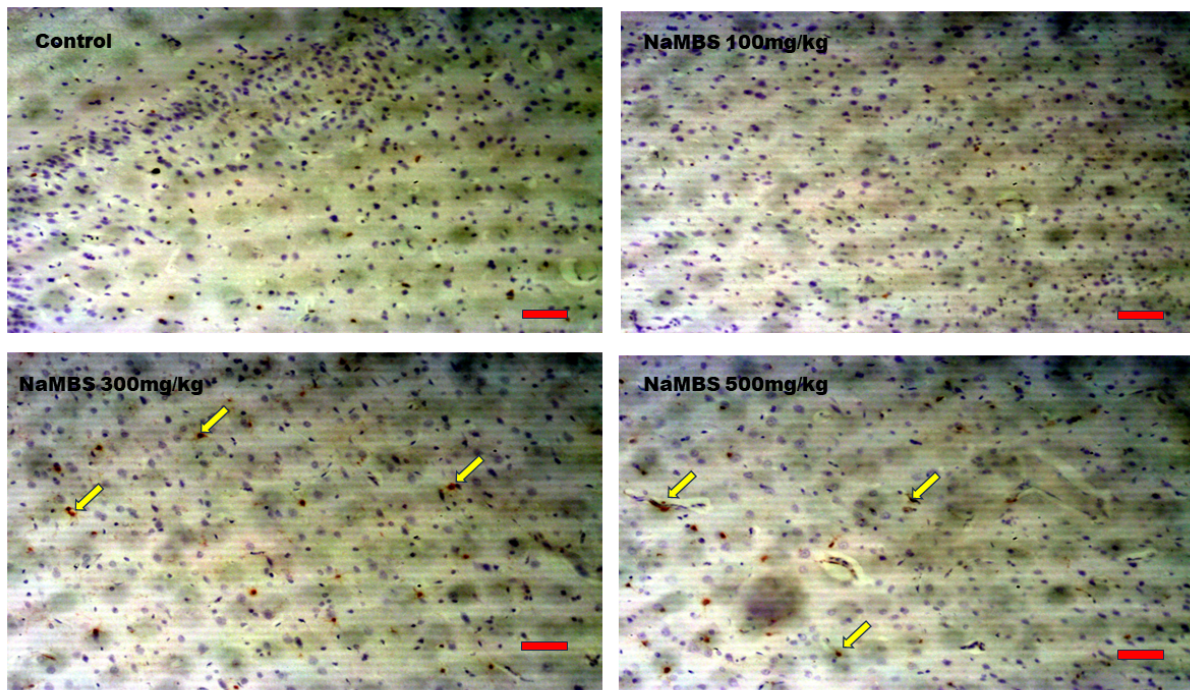


Figure 4. Number of neurons in layer II/III of the prefrontal cortex

NaMBS: Sodium metabisulfite.

Note: Values are presented as Mean±standard deviation.

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Figure 5. Photomicrographs of GFAP-immunostained sections of rat prefrontal cortex of the study groups (magnification $\times 100$)
Abbreviations: NaMBS: Sodium metabisulfite; GFAP: Glial fibrillary acidic protein; PFC: Photomicrographs of prefrontal cortex.
Notes: PFC of the control and 100 mg/kg groups showed no apparent immunoreactivity. PFC of 300 mg/kg and 500 mg/kg groups show minimal immunoreaction (yellow arrows) characterized by star-shaped astrocytes in close contact with neurons.

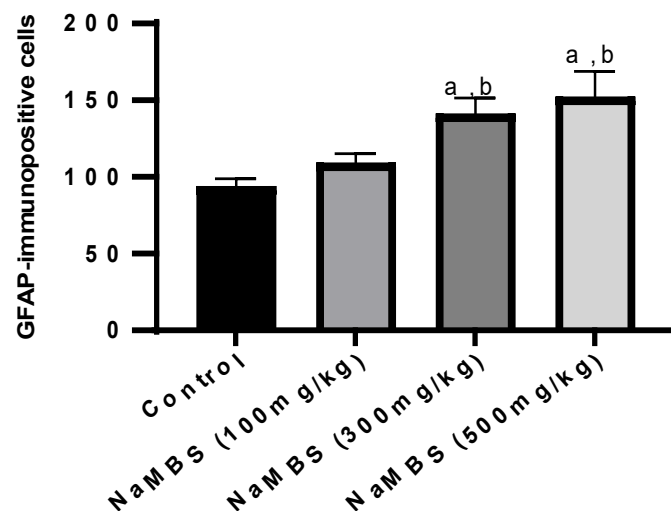


Figure 6. Number of GFAP-immunopositive cells in layer II/III of the prefrontal cortex
NaMBS: Sodium metabisulfite; GFAP: Glial fibrillary acidic protein.

^a $P < 0.05$ vs control; ^b $P < 0.05$ vs NaMBS (100 mg/kg).

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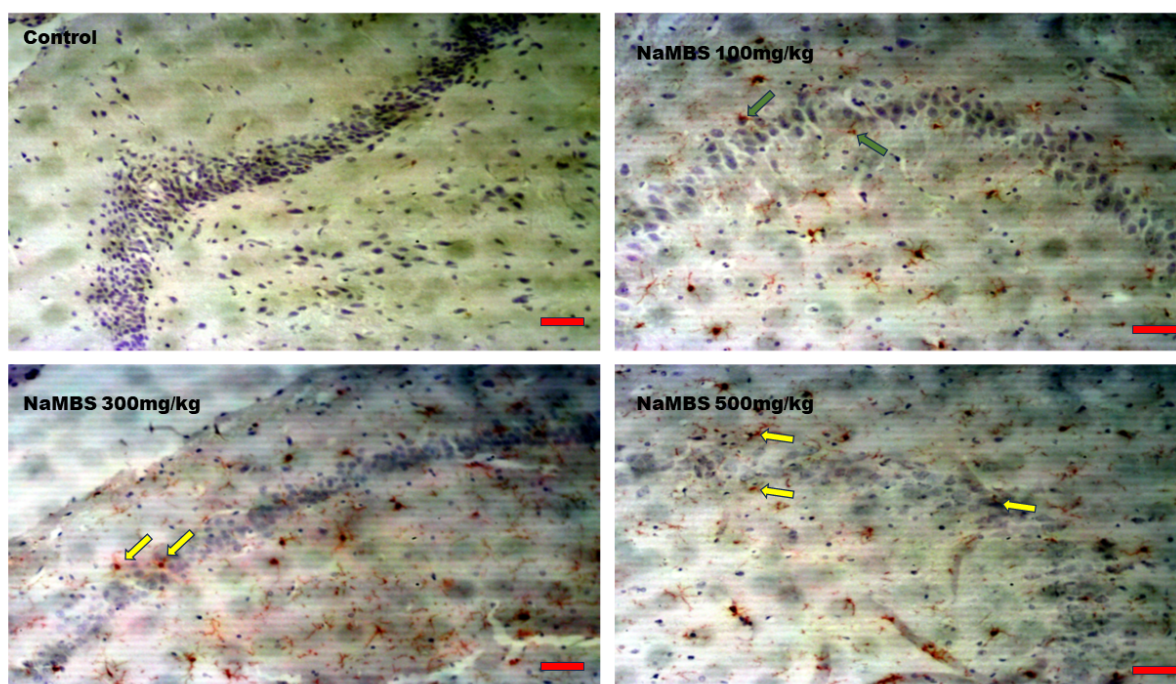

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Figure 7. Photomicrographs of GFAP-immunostained sections of CA1 of rat hippocampus of the study groups (magnification x100)

Abbreviations: NaMBS: Sodium metabisulfite; GFAP: Glial fibrillary acidic protein; CA1: Cornu ammonis.

Notes: CA1 of the control group showed no apparent immunoreactivity of the star-shaped astrocytes in close contact with pyramidal neurons. CA1 of the 100 mg/kg group with minimal immunoreactivity (green arrows). Meanwhile, 300 mg/kg and 500 mg/kg groups showed extensive immunoreaction (red arrows) characterized by star-shaped astrocytes in close contact with pyramidal neurons.

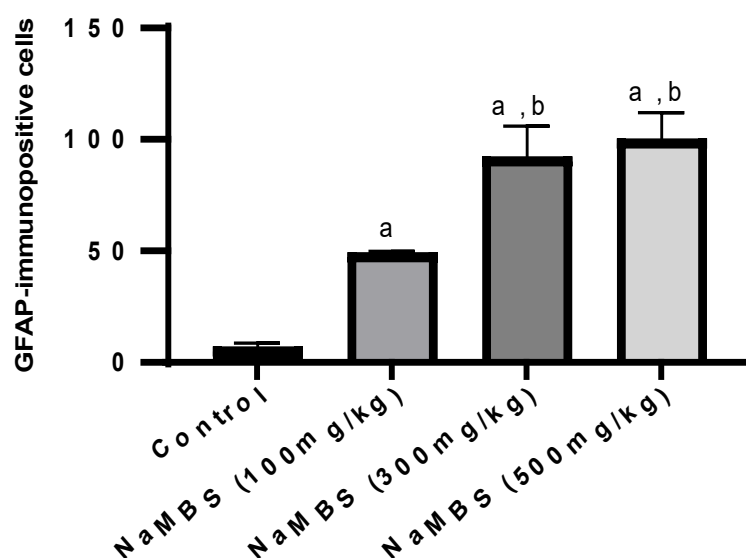


Figure 8. Number of GFAP-immunopositive cells in CA1 of the hippocampus.

NaMBS: Sodium metabisulfite; GFAP: Glial fibrillary acidic protein.

^aP<0.05 vs control, ^b P<0.05 vs NaMBS (100 mg/kg).

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frontal cortex of the NaMBS 300 mg/kg and 500 mg/kg treated groups, there is limited immunoreactivity (indicated by yellow arrows) characterized by astrocytes with star-shaped appearances situated near neurons. Moreover, the quantification of GFAP-immunoreactive cells in the prefrontal cortex (Figure 6) reveals that the number of immunoreactive cells in the NaMBS 300 mg/kg and 500 mg/kg treated groups (140 ± 11 and 151 ± 18 , respectively) was significantly higher ($P < 0.05$) compared to the control group (93 ± 6).

According to Figure 7, the GFAP-immunostained section of CA1 illustrates an absence of evident astrocyte immunoreactivity within the control group. However, the CA1 region of the NaMBS 100 mg/kg treated group displays limited immunoreactivity (green arrows). The NaMBS 300 mg/kg and 500 mg/kg treated groups exhibit pronounced immunoreactivity (red arrows), characterized by a substantial presence of star-shaped astrocytes closely associated with pyramidal neurons. Furthermore, the quantification of GFAP-immunoreactive cells in CA1 (Figure 8) demonstrates a significant increase ($P < 0.05$) in the number of immunoreactive cells across all the treated groups (48 ± 1.5 , 91 ± 15 , and 99 ± 13) compared to the control group (6 ± 2.6).

Discussion

Sodium metabisulfite is a commonly utilized substance for preserving and protecting food, serving as an antioxidant. Nonetheless, there have been documented instances of its negative impacts on the nervous system. The hippocampus is a critical brain region responsible for memory formation, learning, and spatial navigation [21]. The prefrontal cortex, on the other hand, is responsible for planning, decision-making, and social behavior. Research has shown that exposure to toxins can cause cell damage, inflammation, and oxidative stress, leading to impaired function of these brain regions, such as impaired cognitive and learning function [22].

This study has shown that NaMBS 500 mg/kg induces neurodegeneration in CA1 of the hippocampus and affects the number of neurons in the same area. NaMBS enhances sodium currents in rat hippocampal neurons, increasing their excitability and potentially leading to oxidative damage [9]. Also, lipid peroxidation causes excess oxidative stress, which in turn causes cell death [23]. This result indicates that exposure to this compound at 500 mg/kg leads to the progressive loss of neurons or neuronal function in the CA1 area of the hippocampus, which can subsequently result in cognitive and functional consequences associated with hippocampal

dysfunction [24]. The hippocampus plays a crucial role in memory formation and learning, so any damage or degeneration in this region can have significant cognitive and behavioral impacts.

This study has also shown that NaMBS at a dose of 500 mg/kg does not negatively impact the neurons or the overall cellular structure (cytoarchitecture) of the prefrontal cortex. The degeneration of hippocampal neurons under the influence of NaMBS and the lack of noticeable impact in the prefrontal cortex could be due to differences in the expression of sodium channels, the brain's structural redundancy, and its ability to compensate for damage through biochemical and morphological plasticity [9, 25]. Also, research has shown that exposure to the same toxic substance can lead to different patterns of damage or outcomes in other brain areas [25]. In this study, NaMBS elicits increased GFAP-immunoreactivity within the prefrontal cortex. Increased GFAP-immunoreactivity often indicates the activation or response of astrocytes to certain types of brain injury, inflammation, or other neurological changes [26]. The absence of visible effects on neurons and cytoarchitecture, combined with increased immunoreactivity, could imply that the impact of NaMBS on the prefrontal cortex is more subtle and might involve biochemical or molecular changes rather than gross structural alterations.

The Y-Maze spontaneous alternation test is based on the innate tendency of rodents to explore the maze, entering each arm consecutively [15]. This function relies on the ability of their hippocampal cells to fire in complex bursts when they move through specific locations in the environment. This hippocampal-dependent memory is called spatial memory, a form of episodic memory [21]. This study shows no statistical significance in a complete alternation of the rats in the treatment group that received NaMBS compared to rats in the control group. Considering that the Y-Maze Spontaneous alternation test is a test for hippocampal-dependent memory in rodents, the result indicates that hippocampal-dependent memory is not impaired in the NaMBS-exposed group. The preservation of intact memory despite observed damage to neurons in the CA1 area of the hippocampus in this study may be because of the brain's ability to reorganize, rely on alternative pathways, and adapt to damage [27]. Also, compensatory mechanisms, redundancy in neural circuits, and neural plasticity could be reasons behind this observation.

This study shows that the administration of NaMBS at the doses utilized will not significantly affect the MDA concentration. Accordingly, the compound did not in-

duce significant oxidative stress or lipid peroxidation at these specific doses, which is a positive finding as elevated MDA levels are generally associated with cellular damage and dysfunction [28]. While the study did not reveal a significant concentration of MDA, lipid peroxidation may remain elevated. MDA is not a direct indicator of lipid peroxidation and can be influenced by various factors [29]. This potential discrepancy could account for the observed neuronal degeneration in the study.

AchE is a critical enzyme in regulating the neurotransmitter acetylcholine levels, essential for proper nervous system functioning, muscle contraction, and cognitive processes [30]. The inhibition of this enzyme may lead to decreased breakdown and subsequent accumulation of acetylcholine, which could result in excitotoxicity and impaired memory [31]. In this study, the level of AchE in the group treated with NaMBS (500 mg/kg) exhibited a significant reduction compared to the control group. A decrease in the level of AchE suggests that the enzyme's activity is diminished. This could result in an accumulation of acetylcholine, the neurotransmitter responsible for transmitting signals between nerve cells. This reduction in AchE activity could have implications for neurological functions, including excitotoxicity and cognitive activities. Despite the possible accumulation of acetylcholine in the brains of rats in the study, learning and memory activities were unaffected. This is because of the debatable role being played by acetylcholine, as it is believed to be more involved in attention processing than learning and memory [32].

Conclusion

In conclusion, this study sheds light on the complex effects of NaMBS on the nervous system. While it induces neurodegeneration in the hippocampus at a dose of 500 mg/kg, the prefrontal cortex seems resilient to structural changes. Spatial memory remains unaffected, aligned with histological and biochemical analyses. The reduction in acetylcholinesterase activity raises concerns about potential cognitive implications, emphasizing the need for further investigation into the intricate mechanisms underlying NaMBS's impact on neurological function.

Ethical Considerations

Compliance with ethical guidelines

Animals were handled according to the Research Ethics of Adeleke University Ede, Osun State, Nigeria (No.: 00656) in line with the National Institute of Health Guidelines for Care and Use of Laboratory Animals (Publication 1985).

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Conceptualization and Supervision: Nathaniel Ohiemi Amedu; Methodology: Nathaniel Ohiemi Amedu; Investigation and original draft: Favour Wilcox and Habeeb Abdur-Rahman; Review and editing of final draft: All authors; Data collection and Analysis: Nathaniel Ohiemi Amedu, Patrick Abolarin, Favour Wilcox, and Habeeb Abdur-Rahman; Funding acquisition and resources: Nathaniel Ohiemi Amedu and Favour Wilcox.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

The authors take this opportunity to thank Dan Ajibade and Ezra Lambe for their technical support.

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